Application Note
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Detecting Partial Unfolding of Hen Egg White Lysozyme in 2 M Guanidine Hydrochloride Solution using Microfluidic Modulation Spectroscopy (MMS)

- Biosimilars
- mAbs
- ADCs
- AAVs
- Ligand Binding
- ☑ Protein/Peptide Analysis
- VLPs
- Nucleic Acid
- Fusion Proteins
- ☑ Enzyme Analysis

- Aggregation
- Quantitiation
- ☑ Structure
- Stability
- ☑ Similarity

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Introduction

Lysozyme in Guanidine Hydrochloride (GuHCl) has been used as a model system to investigate the impact of denaturants on protein folding and activity using circular dichroism (CD)[1], dynamic light scattering (DLS), and fluorescence[2] spectroscopy. Studies show that GuHCl concentrations of 3-4 M are typically required to induce measurable change in lysozyme structures using these traditional techniques. However, work by Hédoux et al[3] demonstrated that lysozyme actually exhibits a loss of activity at GuHCl concentrations less than 2 M, implying that small changes in secondary structure may occur at lower concentrations of GuHCl but occur below the detection limits of traditional analytical techniques.

Microfluidic Modulation Spectroscopy (MMS) is a novel, ultra-sensitive technique that can be applied using the RedShiftBio Apollo or AQS[3]®pro systems. This technique does not suffer from the masking effects of common excipients and denaturants in its measurements due to real-time background correction provided by rapid modulation of sample and reference buffer streams through the microfluidic flow cell. MMS has demonstrated its ability to see changes in secondary structure and quantify these changes with greater sensitivity and reproducibility than Fourier-Transform Infrared (FTIR) and Circular Dichroism (CD) spectroscopy[4,5].

In this study, MMS was used to confirm that lysozyme undergoes partial unfolding at GuHCl concentrations as low as 0.5 M. The spectra were automatically acquired using a RedShiftBio MMS system powered by delta control software, and processed using the Data Analysis processing engine.

Spectra were processed for Similarity, Area of Overlap[6], and Weighted Spectral Difference (WSD)[7] to confirm that low concentrations of GuHCl induce small but statistically significant changes in secondary structure. Higher order structure analysis was also performed to reveal that the changes were due to an increase in unordered structure and a decrease in turn structures.

Methods

Hen Egg White Lysozyme (HEWL) (Sigma #L6876) was prepared at 10 mg/mL in HPLC-grade water with Guanidine Hydrochloride (Sigma #G3272) concentrations of 0, 0.5, 1, and 2 M. Incubation for all samples occurred for 1 hr at RT. Samples were analyzed in triplicate using MMS at a modulation rate of 1 Hz and 5 psi backing pressure.

Results

I. Raw Differential Absorbance: Figure 1a shows the Raw Differential Absorbance for 0.5, 1, and 2 M GuHCl solutions minus water absorbance (top three traces) and lysozyme solutions in water at three concentrations of GuHCl with the GuHCl and water contributions automatically subtracted (lower three traces). In the lower three lysozyme-GuHCl spectra, the lysozyme signal is a small component of the overall signal relative to the interfering GuHCl signal. To illustrate this, the peak absorbance of 10 mg/mL lysozyme in 2 M GuHCl minus the absorbance of the GuHCl and water was measured and yielded a Raw Diff Absorbance value of 0.058 at a WN of 1656 cm^{-1}. In comparison, the 2 M GuHCl minus the absorbance of water yielded a value of 1.312 at 1656 cm^{-1}. This demonstrates that lysozyme contributed less than 5% of the absorbance signal in the 10 mg/mL lysozyme in 2 M GuHCl solution, and that analysis using MMS can effectively subtract the GuHCl to obtain meaningful data.

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Results, continued

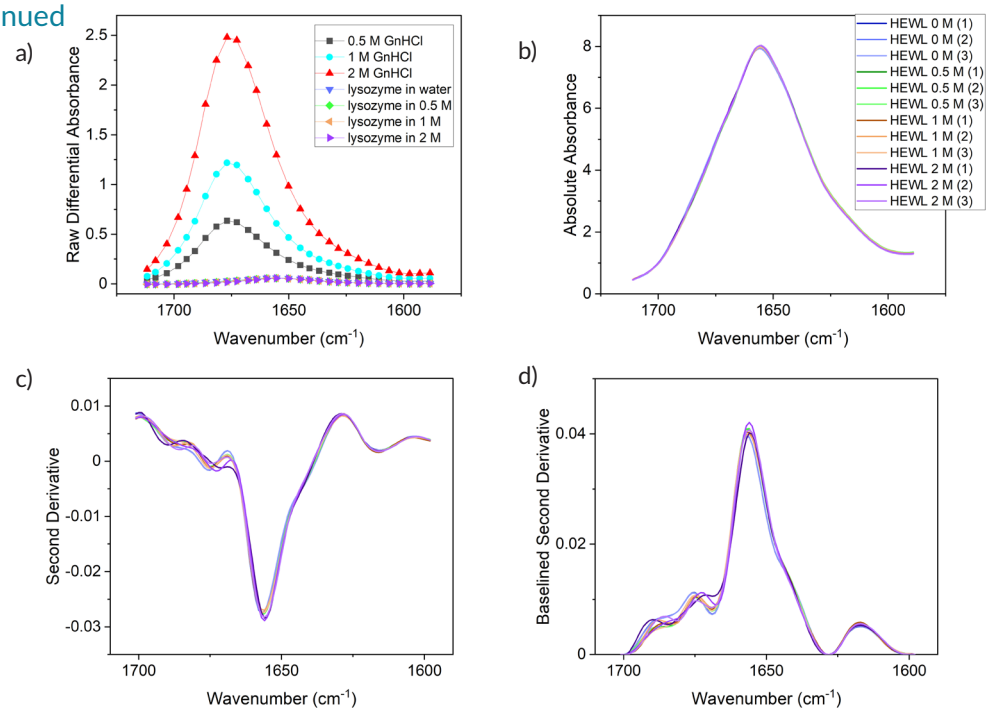

Figure 1: (a) Raw Diff Absorbance of 3 concentrations of GuHCl in water (top 3 traces) and lysozyme in water and GuHCl solutions (lower 3 traces), (b) (c) and (d) 10 mg/mL lysozyme in 0, 0.5, 1, and 2 M GuHCl as Absolute Absorbance, Second Derivative, and Similarity (Area of Overlap) plots, respectively.

II. Absolute Absorbance: The Absolute Absorbance (AbsAU) plot was generated by normalizing the Raw Diff AU for concentration and overlaying all spectra. Figure 1b shows the triplicate Absolute Absorbance spectra of 10 mg/mL lysozyme in 0.5, 1, and 2 M GuHCl. The overlay shows excellent signal-to-noise and high reproducibility across all GuHCl concentrations.

III. Second Derivative: Figure 1c shows the Second Derivative overlay plot generated from the Absolute Absorbance spectra which is useful in highlighting subtle differences that are difficult to discern with the AbsAU overlays alone. This plot shows good overlap across all GuHCl concentrations in the region of 1640 to 1600 cm^{-1} associated with beta-sheet structure. In contrast, the regions of 1700 to 1650 cm^{-1} and 1656 cm^{-1} show greater variance suggesting that GuHCl induces the most significant changes in the turn (1666-1688 cm^{-1}) and unordered (~1650 cm^{-1}) structures, followed by alpha-helix (1656 cm^{-1}) structures, which absorb strongly in this region[8].

IV. Similarity (Area of Overlap): Figure 1d shows the Similarity/Area of Overlap plot for 10 mg/mL lysozyme in 0, 0.5, 1, and 2 M GuHCl. From these spectral results, Area of Overlap and WSD analyses were performed to yield similarity scores and WSD values shown in Table 1.

[GuHCl] (M)	Similarity Score via Area of Overlap		Similarity Score via Weighted Spectral Difference	
	Replicate-to-Replicate	Control-to-Sample	Replicate-to-Replicate	Control-to-Sample
0*	99.89 ± 0.02	99.89 ± 0.02	2.3 E-05	2.3 E-05
0.5	99.81 ± 0.02	97.06 ± 0.11	5.0 E-05	6.3 E-04
1	99.26 ± 0.13	97.01 ± 0.05	1.4 E-04	7.0 E-04
2	98.25 ± 0.37	95.13 ± 0.71	4.3 E-04	1.5 E-03

*Control

Table 1: Similarity Scores and Weighted Spectral Difference for 10 mg/mL lysozyme in 0, 0.5, 1, and 2 M GuHCl relative to the control of 0 M GuHCl (2nd column for both calculations).

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Results, continued

Table 1 shows highly reproducible replicate-to-replicate similarity scores of >98% for lysozyme triplicate analysis at all three GuHCl concentrations, and values of >99% for the 0.5 and 1 M GuHCl concentrations. When 0% GuHCl is used as the control, the control-to-sample similarity score drops to 97% for lysozyme in both 0.5 and 1 M GuHCl. At a GuHCl concentration of 2 M, a much lower similarity value of 95% was observed. In this study, a drop in similarity score to 97% is significant because it is greater than the replicate-to-replicate variance measured of <2%. This similarity analysis confirms that GuHCl induces changes in HEWL secondary structure at a GuHCl concentration as low as 0.5 M. Furthermore, as GuHCl concentration is increased to 2 M, there is an expected additional decrease in similarity indicating continued changes in lysozyme secondary structure.

V. Weighted Spectral Difference (WSD): In addition to the Area Overlap method, similarity was analyzed using Weighted Spectral Difference (WSD) analysis. Also shown in Table I, the WSD values were calculated using the second derivative spectra with 0 M GuHCl as the control. 10 mg/mL lysozyme with 0 M GuHCl exhibits a replicate-to-replicate WSD of 2.3E-05 ± 3.1E-06. Upon addition of GuHCl, the average WSD computes to 6.3E-04, 7E-04, and 1.5E-03 for the 0.5, 1, and 2 M GuHCl solutions, respectively. The WSD values for the 0.5 to 2 M GuHCl samples differ by greater than two standard deviations from the average WSD for the 0 M GuHCl control, again confirming that the addition of GuHCl induces a statistically significant change in the secondary structure that can be detected at a concentration of GuHCl as low as 0.5 M.

VI. Higher Order Structure: While similarity analysis confirmed that lysozyme undergoes a statistically significant change in secondary structure with the addition of as little as 0.5 M GuHCl, it was desired to characterize the nature of the secondary structure changes by structure type. To do this, Gaussian curve-fitting was applied to the Similarity plot to generate the Higher Order Structure plot shown in Figure 2. As expected, the results show that lysozyme is predominantly composed of alpha-helical structures (~42%). At a GuHCl concentration of 0.5 M, there is an observed increase in unordered structures from 10 to 12.5%, and a decrease in turn structures from 27 to 25%. There is less change seen at this concentration for the alpha and beta-sheet structures.

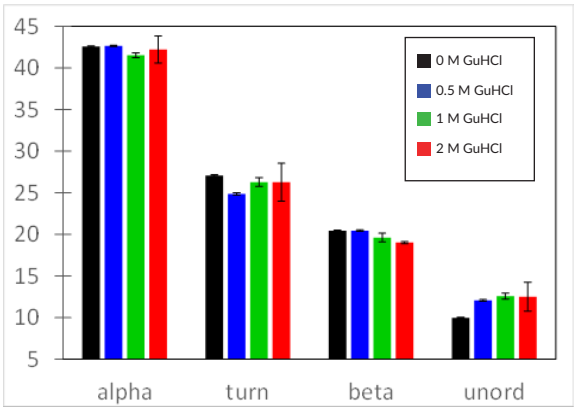

Figure 2: Higher Order Structure plot for 10 mg/mL lysozyme in 0, 0.5, 1, and 2 M GuHCl. Averaged values from 3 replicates are shown for the four secondary structure types.

As the GuHCl concentration was increased to 2 M, MMS measured a gradual increase in unordered structure and slight changes in the remaining structures. The differences seen in the overlay plots for the Similarity/Area of Overlap results for lower concentrations indicate that secondary structure changes actually occur at concentrations of GuHCl as low as 0.5 M in the turn and unordered structures, which have not previously been detected by traditional techniques. Liu et al. confirmed with DLS that the hydrodynamic radius of the molecule did not change[2]; MMS contributed secondary structure information and identified small structural changes that could impact activity[3].

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Conclusions

Acquiring IR measurements in solutions with highly absorbing excipients and denaturants remains a significant challenge in spectroscopy, and it is particularly true of materials, including water, that absorb directly in the spectral region of interest. This study demonstrates that MMS is capable of automatically eliminating the effect of water absorption in the measured IR spectrum as well as minimizing the significant absorbance of the denaturant GuHCl at concentrations as high as 2 M, where GuHCl accounts for >95% of the measured absorbance.

Spectral analysis using MMS for lysozyme in several concentrations of GuHCl resulted in ≥98% replicate-to-replicate reproducibility and therefore allowed statistically significant detection of structural changes to be measured in response to increasing GuHCl concentration. When GuHCl was increased to 2 M, an increase in unordered structures and a change in turn structures was observed across the increasing GuHCl concentration series, and the results at the GuHCl concentration of 0.5 M demonstrate that secondary structure changes occur in lysozyme and can be confidently detected at lower concentrations of GuHCl using MMS where traditional techniques tend to fail.

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